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Original Research

Clinical and genetic drivers of oligo-metastatic disease in colon cancer

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ABSTRACT

Background: Oligo-metastatic disease (OMD) in colon cancer patients exhibits distinct clinical behavior compared to poly-metastatic disease (PMD), with a more responsive and indolent course. This study aims to identify clinical and biological factors uniquely associated with oligo-metastatic behavior.

Methods: Metastatic colon cancer patients from an academic center underwent genetic characterization. OMD was defined as ≤3 lesions per organ, each with a total diameter <70 mm and none exceeding 25 mm. Tumor DNA sequencing by NGS utilized the TruSight Oncology 500 kit. Overall survival (OS) was determined from metastasis diagnosis until death using Kaplan–Meier analysis. Multivariate Cox regression examined prognostic links between clinicopathological and genetic factors. Associations with metastatic patterns were evaluated using Chi-square.

Results: The analysis involved 104 patients (44 with OMD, 60 with PMD). OMD was more prevalent in males ($P = 0.0299$) and with single organ involvement ($P = 0.0226$). Multivariate analysis adjusted for age (>70 vs. <70 years), gender (male vs. female), tumor side (right vs. left), metastatic involvement (more than one site vs. one site), response to first-line therapy (disease control vs. no disease control), and *RAS/BRAF* variants (wild-type vs. mutated) identified OMD vs. PMD as the strongest independent predictor of survival (HR: 0.14; 95 % CI: 0.06-0.33; $P<0.0001$). OMD patients exhibited distinct molecular characteristics, including lower frequencies of *BRAF* p.V600E (P=0.0315) and *KRAS* mutations (P=0.0456), as well as a higher frequency of high tumor mutational

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burden (P=0.0127). Additionally, by integrating data from public datasets and our case study, we hypothesize that some gene alterations (i.e.: *BRAF, SMAD4, RAF1*, and *mTOR*) may prevent OMD occurrence.
Conclusion: OMD, characterized by male predominance, single-site involvement, and distinct molecular features in colon cancer, suggests the need for tailored management strategies.

Introduction

The investigation of oligo-metastatic disease (OMD) in solid tumors has attracted considerable interest in recent years, despite its heterogeneous definition. Specifically, it has been described as the presence of 1–3 metastatic tumors per organ with a size constraint of <7 cm [1]. A more stringent definition includes a maximum of 1–5 lesions with a size limitation of 5 cm [2]. Alternatively, some authors propose defining OMD as metastatic cancer allowing for curative or radical local interventions on all metastatic lesions, regardless of number or volume [3]. Epidemiologically, it is now clear that a subset of patients with metastatic cancer exhibits these characteristics within the spectrum of metastatic disease [4]. From a biological perspective, OMD exhibits distinctive behavior characterized by restricted metastatic dissemination and heightened responsiveness to treatment, clinically evident in the disease indolent course. However, efforts to incorporate the "rate of metastatic growth," typically slower in OMD, into a quantitative criterion remain challenging and less feasible for clinical application [5]. Examining the gradual and indolent clinical progression, juxtaposed with the more aggressive nature of classic poly-metastatic disease (PMD), requires retrospective investigation. Essentially, there is a lack of clear and robust biological and/or molecular factors that uniquely characterize OMD from the outset.

Colorectal cancer (CRC), the focus of the present study, ranks as the third most common cancer, with approximately 1 million deaths from metastatic CRC (mCRC) estimated in 2020 [6]. Unfortunately, about 30-40 % of patients are diagnosed with mCRC, and an additional 30 % will develop it later. The liver is the most typical site of distant spread (about 50 % of patients), followed by non-regional lymph nodes, lungs, and peritoneum. Approximately 10 % of mCRC patients present with OMD at diagnosis [4].

It was previously showed that the *BRAF* p.V600E variant in primary tumors emerged as a potential indicator for excluding oligo-metastatic behavior in mCRC [7]. Moreover, in carefully selected cases of metastatic disease confined to the lungs (e.g., singular lung metastasis not recurring following lung resection over a three-year observation period), unusual genomic trajectories featuring regressive variants in *KRAS* and *SMAD4* from the primary to the metastatic tissues were observed [8]. Exploring genotype-phenotype relationships contributing to CRC progression, with a focus on liver oligo-metastases, evidenced loss of *KRAS* and *SMAD4* alterations and high granzyme-B+ T-cell infiltration associated with non-progressing disease. By contrast, gain in *KRAS, PIK3CA*, and *SMAD4* alterations, along with scarce granzyme-B+ T-cell infiltration, was observed in poly-metastatic spread [9]. Additionally, Next Generation Sequencing (NGS) analysis of liquid biopsies revealed divergent mutational profiles of *KRAS* between primary tumors and metastatic sites. Regressive *KRAS* variants were linked to oligo-metastatic states and favorable survival outcomes, whereas progressive variants were associated with aggressive PMD [10]. The observation of differential cytotoxicity of CD3+/CD8+ lymphocytes against human colon cancer cells carrying different *KRAS* variants in oligo-metastatic CRC patients as well as a higher prevalence of HLA-C7 alleles in oligometastatic CRC patients compared to the general population suggested that the immunological characteristics of tumor/host interactions could significantly influence the trajectory towards oligo-metastatic behavior [11]. One limitation of previous studies was the variability in patient selection and technical methodologies, arising from genetic sequencing performed on diverse NGS platforms.

In this study, we profiled a group of patients diagnosed with both poly- and oligo-metastatic colon cancer at a single institution, utilizing a uniform NGS assay. Our objective was to elucidate and identify the clinical and molecular factors associated with OMD by investigating the clinical characteristics of patients and the genetic features of their primary cancers.

Methods

Study design and primary objective

This retrospective, observational study aimed to discern clinical and/or genetic characteristics that distinguish between oligo- and poly-metastatic behavior in colon cancer.

Patients' selection and clinical management

Patients were recruited between 2016 and 2023 and managed in accordance with ESMO (European Society of Medical Oncology) guidelines [12]. To minimize uncontrollable prognostic confounders, patients with PS ECOG (Performance Status Eastern Cooperative Oncology Group) $\geq$ 2, cachexia risk > 1 [13], and life expectancy < 3 months were excluded. Following consensus discussions among the authors, to avoid heterogeneous and less interpretable results, only patients diagnosed with colon cancer were included. This decision acknowledges the clinical, biological, and molecular differences between colon and rectal cancers. Patients underwent regular monitoring with total body Computed Tomography (tbCT) scan and/or Magnetic Resonance Imaging (MRI) every three months, as indicated, and treatment responses were assessed according to RECIST (Response Evaluation Criteria In Solid Tumours v1.1) [14]. Specifically, disease control (DC) rate is defined as the proportion of patients experiencing complete or partial responses or stable disease relative to the total number of evaluated patients. Lack of disease control corresponds to progressive disease. The study adhered to the principles of the Declaration of Helsinki, and all patients provided written informed consent before receiving any treatments or undergoing genetic assessments. The investigation into genetic determinants of oligo-metastatic colorectal cancer has obtained approval from the competent Ethical Committee ("Comitato Etico IRCCS Pascale") under the "Ricerca Corrente" project (no. L4/8-2022).

Definition of oligo-metastatic disease (OMD)

In this study, OMD is defined as the presentation of metastatic colon cancer with one to three lesions per organ, each with a maximum tumor diameter of <70 mm, and no lesion exceeding 25 mm in diameter. Accurate identification of oligo-metastatic patients for inclusion in the study relies on radiological staging. Additionally, it is crucial to distinguish between two clinical presentations within OMD: those with "oligo-recurrence" and those with "sync-oligometastatic" cases [15]. In the former, the primary tumor has been effectively managed through radical local treatment, while in the latter, OMD coexists synchronously with an active primary tumor. This study has encompassed patients with both presentation types, noting that surgical tumor resection was not performed in only two cases. Finally, patients were classified as oligo-metastatic if they did not exhibit poly-metastatic spread within one year following any local and/or systemic first-line intervention.

Tumor specimens and sequencing

Samples of primary colon cancers were obtained in the form of formalin-fixed and paraffin-embedded (FFPE) tissue specimens, and microdissection of tumor cells was conducted under morphological supervision. DNA isolation was performed using the MGF03-Genomic DNA FFPE One-Step Kit, following the manufacturer's instructions (MagCore Diatech). The quality of DNA was assessed in triplicate using the FFPE QC Kit, also in accordance with the manufacturer's guidelines (Illumina, San Diego, USA). Libraries were prepared using the TruSightOncology 500 kit, targeting the analysis of 523 cancer-related genes (the complete list is provided in **Supplementary File 1**). This assay detects various genomic alterations including small nucleotide variants (SNVs), indels, splice variants, copy number variants, fusions, and immunotherapy biomarkers such as tumor mutational burden (TMB) and microsatellite instability (MSI). Sequencing was conducted on an Illumina NovaSeq 6000 platform (San Diego, USA). TMB was quantified following the method described by Chalmers et al. [16], encompassing all coding somatic base substitutions and indels within the targeted regions, including synonymous alterations. The algorithms for "variant calling" and "TMB calculation" were kept independent to ensure that the number of coding variants was not inferred from TMB, and vice versa (refer to Manufacturer Instructions at https://emea.support.illumina.com/). The size of the targeted (coding) genomic region was 1.9 Mb. MSI, a clinically significant phenotype in colorectal cancers (CRCs), arises from impaired DNA mismatch repair. An accurate exome-based predictive model for MSI phenotype classification was employed. This model utilizes a statistical MSI classifier derived from somatic mutation profiles to distinguish MSI-H (MSI-high) from MSS (MS stable) tumors. The MSI classifier was trained using 999 exome-sequenced TCGA tumor samples with known MSI status (assayed from mononucleotide markers) and achieved a positive predictive value of 98.9 % and a negative predictive value of 98.8 % on an independent test set of 427 samples.

Bioinformatics analysis and data presentation

The bioinformatics pipeline of Illumina TruSight Oncology 500 was utilized for the analysis of sequencing data. A median of 117 million reads were generated per sample, with coverage in the target region exceeding the manufacturer's suggested threshold of 150X. Sequence data were aligned to the human reference genome GRCh37 using the Burrows–Wheeler Aligner with default parameters [17]. Population- and cancer-specific variants were cross-referenced with several databases, including GENCODE, dbNSFP, ICGC-PCAWG, COSMIC, 1000Genomes, ClinVar, CancerMine, OncoScore, CIViC, and CBMDB, to evaluate clinical significance. Variants were filtered using unmatched normal datasets and excluded if the global minor allele frequency was <1 %. Prioritization of variants followed a four-tiered structure (Tier 1-4), in line with AMP/ACMG joint consensus recommendations [18]. Variants with strong clinical significance in cancer were identified based on evidence levels from databases such as CIViC and Cancer Biomarkers.

The prognostic impact of clinic-pathological variables on overall survival (OS) was investigated. OS was calculated from metastatic disease diagnosis until death from colon cancer (cancer-specific survival). Progression-free survival (PFS) was not included due to heterogeneous treatments and radiologic evaluations, making vital status a more reliable outcome. Data were obtained from an electronic database (analyzed in April 2024) containing clinical and pathological information of colon cancer patients treated at the unit of Innovative Therapies for Abdominal Metastases of the Istituto Nazionale Tumori di Napoli "G. Pascale". Covariates were dichotomized for univariate and multivariate analyses: age (>70 vs <70 years), gender (male vs female), tumor side (right vs left), metastatic involvement (more than one site vs one site), response to first-line therapy (disease control vs no disease control), *RAS/BRAF* variants (wild-type vs mutated), and metastatic pattern at diagnosis (OMD vs PMD). OS was assessed using the Kaplan–Meier method, with statistical significance determined by the two-tailed log-rank test for univariate analysis. Multivariate analysis, based on the Cox proportional-hazards regression model, examined prognostic interactions between OS and covariates, with Hazard Ratios (HRs) reported alongside 95 % confidence intervals (CIs). Statistical analyses were conducted using Excel software and MedCalc® version 20.112. Associations between clinicopathological variables and gene alterations were illustrated using contingency tables and assessed using the $\chi 2$ test, with $P < 0.05$ considered statistically significant. We also performed a comparative expression profiling analysis to examine gene expression in TCGA-COAD primary tumor samples compared to controls. We used the online UALCAN program [https://ualcan.path.uab.edu/index.html] [19], which is based on Student's t-test to assess statistical significance between tumor and control groups. Gene expression was considered significant if the p-value was <0.05. Additionally, we evaluated the association of protein-coding genes with patient survival in TCGA-COAD datasets by generating Kaplan-Meier (KM) plots based on gene expression and mutation status. For this purpose, we used online tools including GEPIA2 [http://gepia2.cancer-pku.cn/#index] [20], ENCORI [https://rnasysu.com/encori/panGeneSurvival Exp.php] [21], and cBioPortal [https://www.cbioportal.org/] [22]. Phenolyzer tool was employed to uncover relationships between primary ("seed") genetic variants and secondary ones in the genotypic differences between poly-metastatic and oligo-metastatic patients. Phenolyzer aids in the prioritization and interpretation of genetic variants by querying and linking key gene-disease databases such as OMIM, Orphanet, ClinVar, Gene Reviews, and the GWAS Catalog. It prioritizes genes based on current scientific knowledge, including shared biological pathways, gene family membership, gene-gene transcriptional regulation, and protein-protein interactions. The results are presented through a scoring system, visible at the end of each bar in the specific graph, and a network visualization that offers an intuitive overview of the weighted interaction context (refer to the legend in **Supplementary File 2**). The tool was accessed via the open access site https://phenolyzer.wglab.org/ on June 2024. To study gene interactions, the following parameters were used: disease/phenotype (colon cancer), seed gene interactions (DisGeNET database and Genetic Association Database), and gene scores (Gene Haploinsufficiency Score and Gene Intolerance Score). For a detailed methodology description of this computational tool, refer to Yang et al. [23].

Results

Clinico-pathological characteristics of patients

Two hundred eighty-two metastatic patients were evaluated; however, in accordance with the inclusion criteria, one hundred four were enrolled and analyzed (Fig. 1). Their clinical-pathological characteristics are detailed in Table 1.

Among the patients, 71.2 % were aged <70 years and 28.8 % were >70 years. Analysis within these age groups revealed no statistically significant difference in metastatic patterns, distinguishing between OMD and PMD. Additionally, 34.6 % of the patients were female, and 65.4 % were male. Examination of metastatic patterns based on gender identified a statistically significant difference (P=0.0299), with OMD being more prevalent in males. Sixty-eight point three percent of patients presented with a tumor on the left side of the colon, while 31.7 % presented on the right side. Tumor side did not significantly influence the metastatic pattern (P=0.2088). Overall, G2/G3 grading predominated (93.3 %) compared to G1 (6.7 %), with no significant association revealed between tumor grading and metastatic pattern. The majority of primary tumors presented with pT3 staging (51.9 %). However, the distribution of metastatic patterns did not significantly differ based on pT stage (P=0.3171). Similarly, no significant association was found between pN stage and the occurrence of OMD vs PMD (P=0.4971). Seventy-five percent of patients presented with single organ

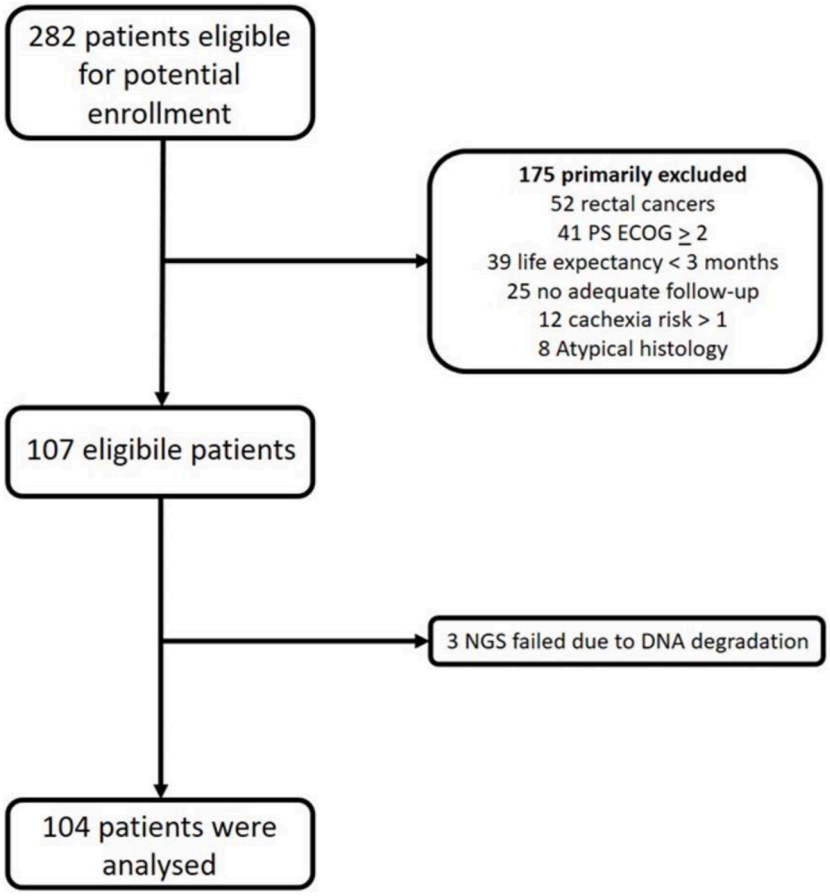

Fig. 1. Flowchart depicting the process of patient inclusion and exclusion in the analyzed cohort.

involvement, and the most frequent single metastatic site was the liver (45 patients). Patients with a single metastatic site exhibited a significantly higher proportion of oligo-metastatic spread (P=0.0226), with 86.4 % having OMD compared to 66.7 % with PMD. Forty-three point three percent of patients underwent adjuvant chemotherapy after surgical removal of primary cancer. There was no significant association between previous adjuvant therapy and metastatic pattern (P=0.4342). A positive response to first-line chemotherapy, a limited number of systemic treatment regimens, the application of stereotactic radiotherapy, or metastasectomies were, as anticipated, more prevalent in OMD. These represent intrinsic characteristics of OMD.

Prognostic effect of OMD and other clinical variables

A comprehensive assessment of prognosis was conducted to enable accurate comparisons between phenotype and genotype. Accordingly, we assessed the prognostic impact of metastatic pattern (OMD vs. PMD) and other clinically relevant variables in the prognosis of metastatic colon cancer through uni- and multi-variate analyses (Table 2).

Age and gender did not significantly affect survival in either analysis, indicating comparable prognosis between patients aged >70 years and those aged <70 years, as well as between males and females. Notably, tumor location significantly influenced survival, with patients with right-sided colon tumors exhibiting shorter median survival (25.0 months) compared to those with left-sided tumors (63.0 months). This association remained significant in the multi-variate analysis (HR: 1.88; CIs: 1.01-3.50; P=0.0455). Variables such as the number of metastatic sites, response to first-line chemotherapy, and *KRAS* mutational status did not show significance in the multi-variate analysis, suggesting that these factors may not independently impact survival beyond the inherent biological characteristics of the disease, which contribute to the differentiation between OMD and PMD. The most noteworthy finding was the distinction between OMD and PMD, where patients with OMD exhibited significantly prolonged median survival (88.9 months) compared to those with PMD (29.0 months). Kaplan-Meier curves depicting the survival of the two groups (OMD vs PMD) subject to genetic analysis are presented in Fig. 2, showing a clear divergence in prognostic trends. This prognostic significance remained highly significant in both uni- and multi-variate analyses (HR: 0.14; CIs: 0.06-0.33; P<0.0001), emphasizing OMD status as a robust and independent positive predictor of survival in patients with metastatic colon cancer.

Genetic characteristics differentiating OMD vs PMD

The two groups, distinguished by their prognostic outcomes (OMD vs. PMD), underwent genetic characterization and analysis to pinpoint specific biomarkers. After reaching consensus among the authors through discussions, our focus shifted towards elucidating point variants and copy number variations in nine specific genes: *BRAF, EGFR, ERBB2, KRAS, MYC, NRAS, PIK3CA, SMAD4*, and *TP53*. As shown in Table 3, only two genes exhibited statistically significant differences: *BRAF* p.V600E and *KRAS* variants.

Notably, compared to patients with PMD, OMD patients displayed a lower frequency of the *BRAF* p.V600E variant (P=0.0315) and *KRAS* variants (P=0.0456). Subsequently, we investigated whether Microsatellite (MS) and Tumor Mutational Burden (TMB) status, two pivotal biomarkers in colon cancer, correlated with different metastatic patterns. Although no statistically significant differences were observed for MS, a noteworthy association was identified regarding the prevalence of high TMB in OMD patients compared to PMD patients (P=0.0127)

Table 1
Clinico-pathological characteristics of analysed patients.

Variable	No.	%	Metastatic pattern		*P*
			Oligo-metastatic No (%)	Poly-metastatic No (%)	
Age					
<70	74	71.2	30 (68.2)	44 (73.3)	
≥70	30	28.8	14 (31.8)	16 (26.7)	0.5686
Gender					
Female	36	34.6	10 (22.7)	26 (43.3)	
Male	68	65.4	34 (77.3)	34 (56.7)	0.0299
Side					
Left	71	68.3	33 (75.0)	38 (63.3)	
Right	33	31.7	11 (25.0)	22 (36.7)	0.2088
Grading					
G1	7	6.7	5 (11.4)	2 (3.3)	
G2/G3	97	93.3	39 (88.6)	58 (96.7)	0.1081
pT					
1/2	13	12.5	6 (14.3)	7 (17.9)	
3	54	51.9	31 (73.8)	23 (58.9)	
4	14	13.5	5 (11.9)	9 (23.1)	0.3171
Unknown	23	22.1	2	21	
pN					
0	30	28.8	17 (40.5)	13 (33.3)	
1	32	30.8	14 (33.3)	18 (46.1)	
2	19	18.3	11 (26.2)	8 (20.5)	0.4971
Unknown	23	22.1	2	21	
No. of metastatic sites					
1 (liver 45; lungs 22; lymphnodes 9; peritoneum 2)	78	75.0	38 (86.4)	40 (66.7)	
≥2	26	25.0	6 (13.6)	20 (33.3)	0.0226
Previous adjuvant therapy					
Yes	45	43.3	21 (47.7)	24 (40.0)	
No	59	56.7	23 (52.3)	36 (60.0)	0.4342
Response to first-line chemotherapy					
DC	73	70.2	28 (93.3)	45 (75.0)	
No DC	17	16.3	2 (6.7)	15 (25.0)	0.0373
No first-line CT	14	13.5	14	0	
No. of systemic treatment lines					
1	15	14.4	13 (43.3)	2 (3.3)	
2	18	17.3	10 (33.3)	8 (13.3)	
>2	57	54.8	7 (23.3)	50 (83.3)	<0.0001
No systemic treatments	14	13.5	14	0	
Stereotactic radiotherapy					
Yes	13	12.5	9 (20.5)	4 (6.7)	
No	91	87.5	35 (79.5)	56 (93.3)	0.0366
Liver or lung metastasectomy					
Yes	22	21.2	18 (40.9)	4 (6.7)	
No	82	78.8	26 (59.1)	56 (93.3)	<0.0001

(Table 4).

To enrich our understanding of genotype/phenotype correlations and to generate hypotheses about specific genes involvement, we scrutinized all genes exhibiting Tier 1-3 alterations. Emphasis was placed on delineating prognostic disparities across the metastatic spectrum. Thus, for descriptive purposes only, without attempting statistical associations, we categorized patients into three groups: those with PMD surviving <30 months (18 patients), oligo-metastatic patients with survival rates akin to poly-metastatic patients (<36 months, 6 patients), and oligo-metastatic patients surviving beyond 5 years of follow-up (15 patients). We chose to highlight only genes altered in at least 2 patients to aid their description and uphold biological relevance. Despite the purely descriptive nature of the analysis, the absence of *SMAD4* alterations in OMD patients warrants attention (Table 5).

However, to provide potential genes involved in the determination of the OMD, we prioritized all genes whose mutation is present only in patients with PMD and/or OMD with poor prognosis (dead <36 months) in our cohort. An additional criterion was based on their biological association with the poly-metastatic disease, thereby excluding an oligo-metastatic behavior. In order to do this we analyzed public datasets (see Methods). Based on these criteria, we selected the following genes: *BCL6, BRAF, FAT1, mTOR, NKX2-1, RAF1*, and *SMAD4* (Table 6). According to Phenolyzer tool, the most important and interrelated genes were: *BRAF, SMAD4, RAF1* and *mTOR* (Fig. 3).

Discussion

The scientific community interest in investigating the biological determinants of oligo-metastatic behavior is steadily increasing. This is because the identification of robust biomarkers can facilitate early recognition, enabling more effective treatments while sparing the toxicity of unjustified therapies. Therefore, identifying the clinical and biological characteristics of OMD is a priority in biomedical research.

In our study, several findings deserve emphasis and discussion. Some are clinical, such as the gender-based disparity favoring males and the observation that oligo-metastatic behavior is more prevalent when only one organ is involved. Other factors are genetic and require genetic profiling using NGS for detection. Specifically, OMD is more common when the primary tumor lacks *KRAS* and/or *BRAF* p.V600E variants. The biological explanation of this finding lies in the role that these variants play in stimulating proliferation and metastatic spreading. *KRAS* encodes a GTPase involved in various cellular signal transduction pathways that regulate cell proliferation, differentiation, and survival [24]. The involvement of *KRAS* variants in colorectal cancer is well-established, with approximately 40 % of metastatic CRC cases harboring *KRAS* variants [25]. Oncogenic *KRAS* variants play a pivotal role in progression by constitutively activating downstream signaling cascades, particularly the RAF-MEK-ERK pathway, promoting uncontrolled cell growth, tumor development, and poor prognosis [26]. BRAF is a serine/threonine kinase involved in the mitogen-activated protein kinase (MAPK) signaling pathway, regulating cell growth, proliferation, and differentiation [27]. In CRC, the most common *BRAF* variant is the V600E substitution, leading to constitutive activation of the MAPK

Table 2
Uni- and multi-variate analysis of prognostic power of clinical characteristics in metastatic CRC patients.

Co-variate	Dicothomization	Median survivals (months)	No. of events/patients	*P* at univariate	HR	95 % CI	***P* at multivariate**
Age	≥70y vs <70y	54.0 vs 47.0	16/30 vs 35/74	0.7042	1.86	0.99-3.49	0.0512
Gender	M *vs* F	42.0 vs 54.0	35/68 vs 16/36	0.6079	1.58	0.82-3.03	0.1636
Side	R vs L	25.0 vs 63.0	20/33 vs 31/71	0.0200	1.88	1.01-3.50	0.0455
No. of metastatic sites	>1 vs 1 site	32.0 vs 80.0	34/78 vs 17/26	0.0083	0.77	0.40-1.48	0.4429
Response to first-line CT	DC *vs* no DC	69.0 vs 29.0	35/81 vs 16/23	0.0142	0.60	0.31-1.17	0.1380
***KRAS* mutations**	Mutated vs wild-type	32.0 vs 87.0	25/40 vs 26/64	0.0135	1.72	0.93-3.16	0.0808
Oligo- vs poly-metastatic disease	Yes vs No	88.0 vs 29.0	16/44 vs 35/60	<0.0001	0.14	0.06-0.33	<0.0001

CI: Confidence Interval; DC: Disease Control; F: Female; HR: Hazard Ratio; L: Left; M: Male; R: Right.

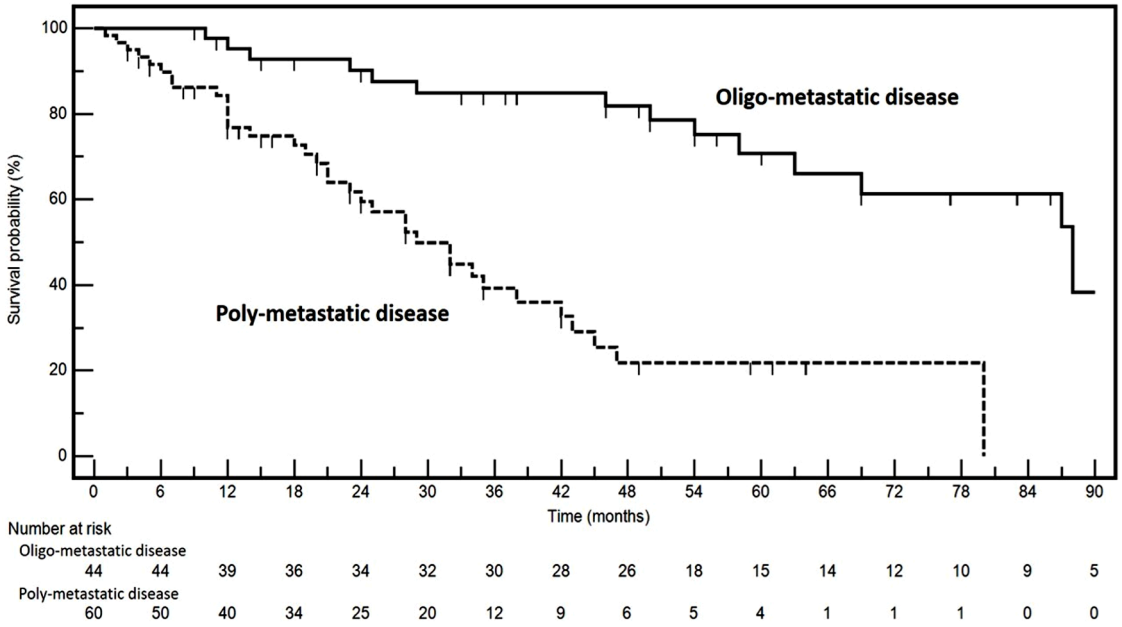

Fig. 2. Kaplan-Meier survival curves, stratified by metastatic pattern as defined in the Methods section, demonstrate distinct outcomes. Patients with oligo-metastatic disease exhibit clear divergence in survival curves compared to those with poly-metastatic disease, with median survivals of 88.0 versus 29.0 months, respectively (P<0.0001 by Log Rank test). The number at risk is reported below the figure.

Table 3
Associations between genetic alterations in the analyzed series and metastatic patterns (oligo- vs poly-metastatic).

Gene	Metastatic pattern		*P*
	Oligo-metastatic No (%)	Poly-metastatic No (%)	
BRAF			
p.V600E	0 (0.0)	6 (10.0)	
Wild-type	44 (100.0)	54 (90.0)	0.0315
EGFR			
Mutated	0 (0.0)	1 (1.7)	
Copy number gain	7 (15.9)	6 (10.0)	
Wild-type	37 (84.1)	53 (88.3)	0.4735
ERBB2			
Mutated	10 (22.7)	18 (30.0)	
Copy number gain	6 (13.6)	9 (15.0)	
Wild-type	28 (63.6)	33 (55.0)	0.6524
KRAS			
Mutated	12 (27.3)	28 (46.7)	
Wild-type	32 (72.7)	32 (53.3)	0.0456
MYC			
Mutated	3 (6.8)	3 (5.0)	
Copy number gain	11 (25.0)	19 (31.7)	
Wild-type	30 (68.2)	38 (63.3)	0.7306
NRAS			
Mutated	1 (2.3)	7 (11.7)	
Wild-type	43 (97.7)	53 (88.3)	0.0771
PIK3CA			
Mutated	7 (15.9)	10 (16.7)	
Wild-type	37 (84.1)	50 (83.3)	0.9182
SMAD4			
Mutated	7 (15.9)	12 (20.0)	
Wild-type	37 (84.1)	48 (80.0)	0.5955
TP53			
Mutated	31 (70.5)	34 (56.7)	
Wild-type	13 (29.5)	26 (43.3)	0.1533

Table 4
Metastatic patterns (oligo- vs poly-metastases) in relation to micro-satellite (MS) status and tumor mutational burden (TMB).

	Metastatic pattern		*P*
	Oligo-metastatic No. (%)	Poly-metastatic No (%)	
MS			
Stable	5 (11.4)	2 (3.3)	
Unstable	39 (88.6)	58 (96.7)	0.1081
TMB			
<10	29 (65.9)	47 (78.3)	
>=10 <20	9 (20.5)	13 (21.7)	
≥=20	6 (13.6)	0 (0.0)	0.0127

pathway and promoting oncogenesis [28]. *BRAF* variants occur in approximately 5-15 % of CRC cases, often mutually exclusive with *KRAS* variants. They are associated with distinct clinic-pathological features, including older age at diagnosis, female predominance, right-sided tumor location, and poorer prognosis compared to *BRAF* wild-type tumors [29,30].

Despite the limitation imposed by the small sample size, an intriguing and unexpected finding was the absence of *SMAD4* variants in primary CRC cases progressing to long-term survival and OMD. SMAD4 functions as a downstream effector of the transforming growth factor (TGF)-β pathway. Briefly, upon ligand binding, TGF-β receptor 2 phosphorylates TGF-β receptor 1, which subsequently phosphorylates downstream proteins SMAD2 and 3, initiating the "canonical" signaling pathway [31,32]. SMAD2 and 3 form a complex with SMAD4 and translocate into the nucleus, where they activate transcription of numerous target genes, including *SERPINE1, LTBP2, CDKN1A, ARID3B, ATXN1, PTPRK, RAB6A, SMAD7, EHBP1*, among others, predominantly acting as tumor-suppressor genes in TGF-β-mediated signaling [33]. SMAD4 alterations plays a critical role in the malignant phenotype of poly-metastatic CRC and serves as an independent negative prognostic factor for disease-free and overall survival in advanced CRC [34–36].

In addition to *KRAS, BRAF* and *SMAD4*, other genes were hypothesized to influence the metastatic potential of our oligo-metastatic series. Among them *BCL6* has been linked to tumorigenesis through its ability to inhibit p53-dependent apoptosis, thereby promoting cell survival and proliferation under oncogenic stress [37]. *FAT1*, a member of the cadherin superfamily, functions as a tumor suppressor by modulating Wnt

Table 5
Descriptive analysis of gene alteration concordance in poly- and oligo-metastatic patients with extreme survival outcomes.

	Poly-metastatic patients	Oligo-metastatic patients	
	Dead <30 months (18)	Dead <36 months (6 pts)	Alive >5 years (15 pts)
AKT1	2	0	0
AMER1	0	0	2
APC	7	3	10
ARID1A	0	0	2
ARID1B	2	0	0
ARID2	0	2	0
ATM	0	0	2
BARD1	7	5	8
BCL6	2	0	0
BRAF	3	0	0
CCND3	4	0	0
CREBBP	0	0	2
EP300	0	0	2
ERBB4	0	0	2
FAT1	4	2	0
FBXW7	2	3	2
FGFR4	4	3	0
KAT6A	2	2	0
KDR	3	0	2
KRAS	10	3	4
LRP1B	3	2	3
MTOR	2	0	0
NKX2-1	0	2	0
NOTCH1	2	0	0
NRAS	0	0	2
PIK3CA	2	0	3
PTEN	2	0	0
PTPRT	0	0	3
RAF1	2	0	0
RBM10	2	0	0
REL	0	0	2
RNF43	2	0	0
ROS1	2	0	0
SLIT2	0	2	0
SMAD4	4	3	0
SMO	0	2	0
TP53	10	4	10
TSC2	0	0	2
ZFHX3	0	2	2
ZNF217	2	0	0

signaling and maintaining cell-cell adhesion. Its alterations in colorectal cancer are associated with increased cell migration, invasion, and metastasis [38]. *MTOR*, the mechanistic target of rapamycin, is a central regulator of cell growth, proliferation, and survival, responding to nutrient availability and cellular energy status. Aberrant activation of the mTOR pathway is frequently observed in colorectal cancer, where it promotes tumor growth and metastasis [39]. *NKX2-1*, also known as thyroid transcription factor-1 (TTF-1), which is typically associated with lung and thyroid tissues, has emerging implications in colorectal cancer. Although its role is less well-defined compared to other genes, recent studies suggest that *NKX2-1* may contribute to colorectal tumorigenesis through its involvement in cellular differentiation and proliferation pathways [40]. Finally, *RAF1*, another member of the RAF kinase family, operates within the same MAPK/ERK pathway as BRAF. Mutations and dysregulation of *RAF1* can lead to uncontrolled cell proliferation and survival, contributing to colorectal cancer development [41]. Collectively, these genes represent critical components of the molecular landscape of colorectal cancer, each contributing to the disease's complexity through various mechanisms. Thus, the oligo-metastatic phenotype may arise from a combination of these complex genetic features.

An exceptionally interesting observation, potentially guiding further investigation into the tumor-host immune system relationship, is the higher incidence of high tumor mutational burden (TMB) in OMD patients. TMB reflects the number of nonsynonymous mutations in tumor cell genomes and has been prospectively validated as a positive biomarker predicting response to immunotherapy [42,43]. A high TMB is likely to increase the generation of novel tumor antigens ("neo--antigens"), rendering cancer cells immunogenic. It is plausible that in metastatic patients, exposure to numerous tumor neo-antigens enhances recognition by the patient's immune system, leading to better tumor control and directing clinical evolution towards OMD. Our previous studies indicated that loss of *KRAS* and *SMAD4* alterations from primary to metastatic lesions was associated with oligo-metastatic behavior, and this genetic regression correlated with high granzyme-B+ T-cell infiltration into metastatic tumors [9,10]. The propensity to accumulate mutations is driven by defects in DNA repair mechanisms, which in some cases are associated with microsatellite instability [44].

Interestingly, high TMB and microsatellite instability (MSI) are not equivalent from biological or clinical perspectives. High TMB is typically defined as ≥10 mutations per megabase [45]; however, in our study, we additionally divided TMB values into ≥10 <20 and ≥20. The cut-off of ≥20 mutations per megabase has been used in several immunotherapy studies. Such heterogeneity documents a substantial lack of standardization in this the field [46,47]. Although we did not generate direct immunologic data in this regard, the association between TMB and OMD emerges for the first time in scientific literature and is intriguing, representing an indirect evidence that the oligo-metastatic behavior may depend not only on individual genes but also on the quality of tumor-host immunologic interactions.

It is important to acknowledge and discuss several limitations of our study. Firstly, the study is retrospective in nature and includes a consecutive yet relatively small sample size of patients. Despite these limitations, the single-center design and consistent technical approach to genetic evaluations help to mitigate these concerns. Moreover, the selection criteria employed (PS ECOG 0 or 1 patients) are designed to mitigate the prognostic influence of potential confounding factors, which are frequently intricate and difficult to ascertain, primarily pertaining to patients' overall clinical status. This approach also serves to mitigate biases inherent in the retrospective design of the study. The retrospective analysis remains necessary for defining OMD since some patients initially diagnosed as OMD may progress to develop PMD within one year of treatment, thus being "false" OMD.

In conclusion, this study contributes to define the clinical and genetic characteristics guiding the identification of true OMD when a patient has this onset in clinical practice. From our data it can be suggested that the following clinical and molecular characteristics can be associated to the development of a OMD: sex (male), involvement of a single organ, primary tumor lacking *KRAS* and/or *BRAF* p.V600E variants, and low TMB. Other emerging genes such as *BRAF, SMAD4, RAF1* and *mTOR* could be also involved in the determination of the poly-metastatic disease and deserve additional evaluation in larger series of patients. Identifying true oligo-metastatic behavior can have significant prognostic and therapeutic implications, avoiding overtreatment and favoring local and/or innovative treatments for these patients.

Declarations

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Institutional review board statement

The study adhered to the principles of the Declaration of Helsinki, and all patients provided written informed consent before receiving any treatments or undergoing genetic assessments. The investigation into

Table 6
Prognostic impact of genes mutated exclusively in poly- or oligo-metastatic disease.

Gene	TPM [Primary tumor vs Normal]	Statistical significance [Primary tumor vs Normal]	Expression status [Primary tumor vs Normal]	Log rank p-value * (for altered expression)	Interpretation	Log rank p-value* (for gene mutation)	Interpretation
AKT1	111.973	1.29E-01	Upregulated	0.46	NS	0.924	NS
AMER1	2.585	1.11E-16	Significantly upregulated	0.9	NS	0.357	NS
ARID1A	39.23	4.46E-01	Downregulated	0.12	NS	0.0112	The presence of mutations worsens the prognosis.
ARID1B	14.516	2.02E-03	Significantly upregulated	0.88	NS	0.0918	NS
ARID2	5.844	4.76E-04	Significantly upregulated	0.55	NS	0.199	NS
BCL6[A]	6.305	9.08E-01	Downregulated	0.048	The high expression worsens the prognosis.	0.045	The presence of mutations worsens the prognosis.
BRAF[A]	2.824	1.64E-07	Significantly upregulated	0.9	NS	3.55E-04	The presence of mutations worsens the prognosis.
CCND3	46.675	2.26E-06	Significantly downregulated	0.52	NS	0.66	NS
CREBBP	15.188	7.32E-01	Downregulated	0.45	NS	0.035	The presence of mutations worsens the prognosis.
EP300	18.064	1.07E-01	Downregulated	0.86	NS	0.755	NS
ERBB4	*	3.84E-04	Significantly downregulated	0.08	NS	0.912	NS
FAT1[A]	150.227	1.11E-16	Significantly upregulated	0.37	NS	6.39E-03	The presence of mutations worsens the prognosis.
FGFR4	55.7	<1E-12	Significantly upregulated	0.6	NS	0.414	NS
KAT6A	10.02	1.62E-03	Significantly upregulated	0.39	NS	0.414	NS
MTOR[A]	17.406	4.93E-01	Downregulated	0.84	NS	0.047	The presence of mutations worsens the prognosis.
NKX2-1[A]	*	3.74E-03	Significantly upregulated	0.99	NS	0.034	The presence of mutations worsens the prognosis.
NOTCH1	21.463	6.21E-07	Significantly upregulated	0.43	NS	0.098	NS
NRAS	29.295	1.47E-01	Downregulated	0.32	NS	0.024	The presence of mutations worsens the prognosis.
PTEN	17.67	1.66E-12	Significantly downregulated	0.8	NS	0.865	NS
PTPRT	0.014	1.99E-13	Significantly downregulated	1	NS	0.016	The presence of mutations worsens the prognosis.
RAF1[A]	45.433	2.32E-12	Significantly downregulated	0.53	NS	0.031	The presence of mutations worsens the prognosis.
RBM10	40.761	9.84E-07	Significantly upregulated	0.56	NS	0.549	NS
REL	2.128	1.34E-01	Upregulated	0.58	NS	0.098	NS
RNF43	109.468	1.62E-12	Significantly upregulated	0.24	NS	0.962	NS
ROS1	0.011	9.22E-02	Downregulated	0.78	NS	0.87	NS
SLIT2	0.366	6.81E-04	Significantly downregulated	0.11	NS	0.802	NS
SAMD4[A]	2.946	3.50E-03	Significantly downregulated	0.017	The high expression worsens the prognosis.	0.683	NS
SMO	4.095	2.41E-04	Significantly upregulated	0.8	NS	0.092	NS
TSC2	41.538	3.95E-04	Significantly upregulated	0.76	NS	0.016	The presence of mutations worsens the prognosis.
ZNF217	20.5	8.88E-01	Downregulated	0.94	NS	0.193	NS

[A] Mutations in these genes or their elevated expression in tumor tissue compared to normal tissue are absent in our cases of oligo-metastatic disease and are associated with a worse prognosis in public datasets.

* The overall survival analysis based on gene expression/mutation was performed using GEPIA2. However, for the genes *ERBB4* and *NKX2-1*, no data were available in GEPIA2; therefore, the OS plots for these two genes were generated using ENCORI.

NS: Not significant

genetic determinants of metastatic colorectal cancer has obtained approval from the Ethical Committee of University of Campania "Luigi Vanvitelli" with the numbers 790 and 68 for project i-Cure.

Data availability statement

Authors are willing to provide the sequencing data, which is subject to privacy concerns, upon a reasonable request to giovanni.sav arese@centroames.it.

CRediT authorship contribution statement

Alessandro Ottaiano: Conceptualization, Methodology, Resources, Supervision, Validation, Writing – original draft. **Mariachiara Santorsola:** Conceptualization, Methodology, Writing – original draft. **Roberto Sirica:** Conceptualization, Formal analysis, Methodology,

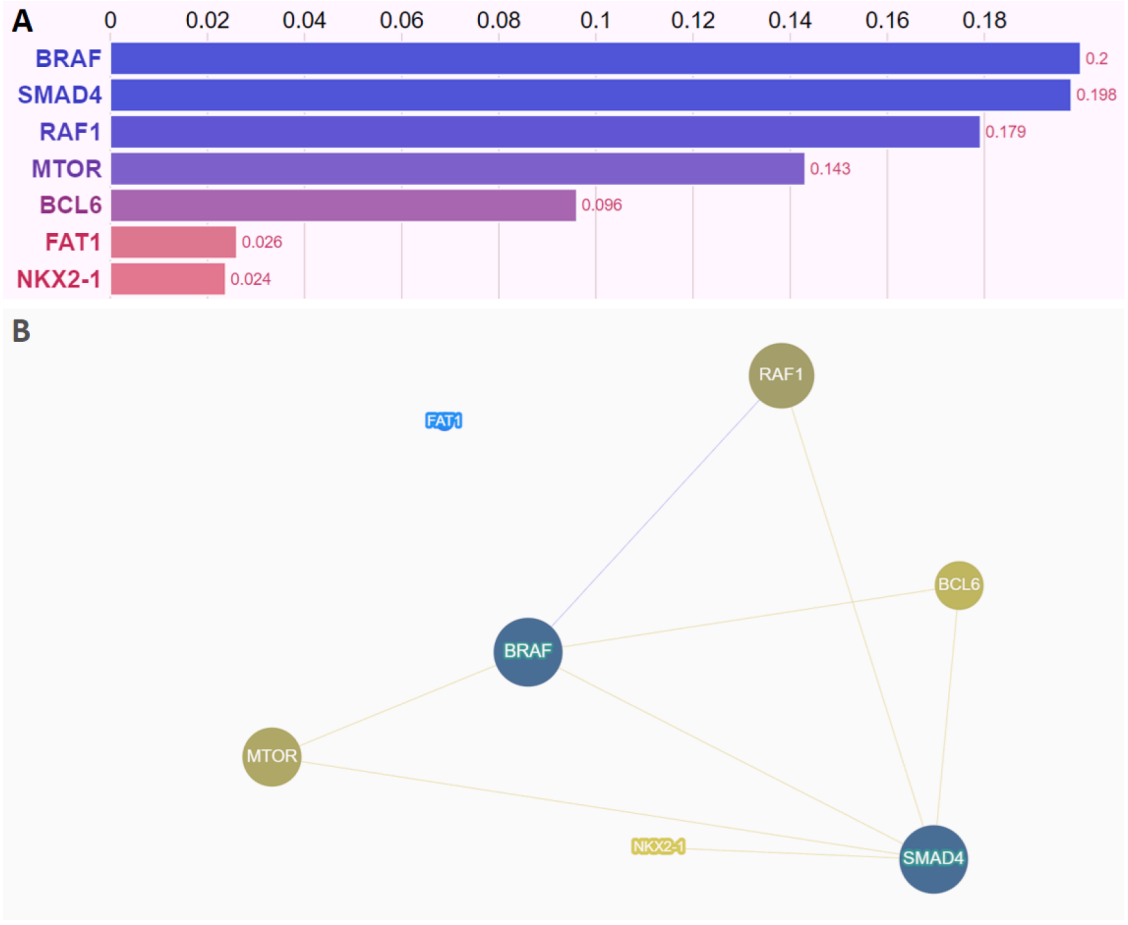

Fig. 3. Prioritization of genes involved in worsening the prognosis of colon cancer and being not altered in oligo-metastatic patients was performed using the Phenolyzer tool. In the upper section (A), the Phenolyzer score, ranging from 0 to 1, is displayed. A higher score indicates a stronger association between the gene and the disease. The lower panel (B) features a network visualization tool that illustrates gene–gene and gene–disease relationships (see Materials and Methods for further details).

Software, Validation, Writing – original draft. **Annabella Di Mauro:** Investigation. **Antonella Di Carlo:** Investigation. **Monica Ianniello:** Investigation. **Francesco Sabbatino:** Data curation. **Rosa Castiello:** Investigation. **Francesca Del Peschio:** Investigation. **Marco Cascella:** Data curation, Writing – review & editing. **Francesco Perri:** Data curation. **Maurizio Capuozzo:** Formal analysis. **Nicola Martucci:** Data curation. **Edoardo Mercadante:** Data curation. **Valentina Borzillo:** Data curation. **Rossella Di Franco:** Data curation. **Francesco Izzo:** Data curation. **Vincenza Granata:** Investigation. **Carmine Picone:** Investigation. **Antonella Petrillo:** Investigation. **Massimiliano Berretta:** Supervision, Validation. **Salvatore Stilo:** Investigation. **Luca Tarotto:** Investigation. **Anna Chiara Carratù:** Data curation. **Gerardo Ferrara:** Data curation. **Madhura Tathode:** Formal analysis, Validation. **Alessia Maria Cossu:** Validation. **Marco Bocchetti:** Writing – review & editing. **Michele Caraglia:** Formal analysis, Methodology. **Guglielmo Nasti:** Supervision, Writing – review & editing. **Giovanni Savarese:** Conceptualization, Investigation, Resources, Software, Supervision.

Declaration of competing interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in the paper entitled “Clinical and genetic drivers of oligo-metastatic disease in colon cancer.”

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Supplementary materials

Supplementary material associated with this article can be found, in the online version, at doi:10.1016/j.neo.2024.101111.

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